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Immune Response of Rats, Rabbits and Chickens Challenged with Sheep Erythrocytes and Pretreated with *Capparis Spinosa* and Cyclophosphamide

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Abstract : Humoral and cell mediated immune response of methanol extract of *Capparis spinosa* (*C. spinosa*) and cyclophosphamide (CY) was the aim of this study. Rats, rabbits and chickens were selected for assessment. Each species was divided into three groups: first group was challenged with sheep erythrocytes (SRBCs) at days zero and 15, second group was challenged after 3 days of CY injection and the last group was challenged after two weeks of *C. spinosa* extract administration. The results of this study showed that, in comparison to challenged group, total leukocytic count (TLC) and spleen cellular viability % was significantly decreased in CY treated animals ($P<0.05$). Neutrophils (N%) and lymphocytes **(**L%) were significantly lowered at days zero, 14 and 42 for rat, at days zero and 14 for rabbits and at days 14 and 42 for chickens ($P<0.05$). At day 42, TLC and L% were significantly decreased in rats and rabbits pretreated with *C. spinosa*. Rabbits pretreated with *C. spinosa* showed a significant ($P<0.05$) higher N% at days zero and 14. A significant increase of H% was obtained in chickens pretreated with *C. spinosa*. In contrast to challenged rats and rabbits, the phagocytic index and glucose consumption of lymphocytes of CY pretreated group was significantly decreased ($P<0.05$), while it was elevated in *C. spinosa* pretreated rats and chickens ($P\leq0.05$). It can be concluded that pretreatment of animals with *C. Spinosa* extract enhanced the immune system. Therefore, the results of this study may encourage the use of *C. spinosa* extract in reducing the immune depression of CY in animal models.
Key words : *Capparis spinose;* Cyclophosphamide; Immune responses; Phagocytic index.

Introduction

The effectiveness of traditional plants with low side effects and cost encouraged peoples in Saudi Arabia to use it in treating many immune diseases. However, the mechanism of most of these herbs have not been defined precisely. Large scale production of honey from *Capparis spinosa* (*C. spinosa* - Shefallah) in Saudi Arabia[1] encouraged us to investigate the humoral and cellular immune responses of methanolic extract of *C. spinosa* in comparison to a negative response of CY. Rat, rabbit and chicken were selected for assessment.

C. spinosa is native to the Mediterranean region and grow wild in desert areas including Saudi Arabia and India[2]. Previously, it was used in some populations as a carminative, diuretics, astringents, and tonic[3]. *C. spinosa* improved the levels and immune activity of mononuclear cellsagainst virus infection by expressing some proinflammatory cytokines [4]. Manikandaselvi et al.[2] summarized in table the effect of *C. spinosa* as anthelminthic[5], anti-cytotoxic activity[6], anti-inflammatory[7], antiarthritic[8], antioxidant[9], antibacterial[10],

antidiabetic[11], immunomodulatory[12], anticarcinogenic[13] and anti-hepatotoxic activity [14]. The phytochemicals identified in this plant were lipids, alkaloids, glucocapperin, and polyphenols[15].

CY as a cytotoxic drug was previously used as chemotherapeutic agent and immunosuppressive therapy against cancer[16]. The mechanism of CY in inducing immunosuppression has been reported[17]**.** CY was able to bind to DNA specifically to the N7 of guanine residue and forms double strand ducts[17]. At cellular level, it leads to apoptosis and induces cytotoxic effect on murine lymphocytes[18]. The drug also causes generation of reactive oxygen radicals[17]. In addition, CY produces acrolein, secondary metabolite to inhibit the cell proliferation, and also modulates the expression levels of gene sand transcription factors and lowers the activator protein-1[19].

Blood samples were taken prior to challenge with SRBCs or on the fourth day of CY injection or after two weeks of *C. spinosa* extract administration (day 0) and on days 14 and 42 post-challenge. Part of blood samples were used for counting TLC as well as N %, L % and N/L ratio within one hour after collection. The solid phase enzyme-linked immunosorbent assay (ELISA) using membrane of SRBCs as antigen was performed to estimate total serum Igs, IgM and IgG at days 0 (pre-challenge), 7, 14, 21, 28, 35 and 42 (post-challenge). Spleen cellular viability % and histopathology of spleen were also evaluated. Cellular immune response was performed by calculation of phagocytosis index using *in vivo* and *in vitro* carbon clearance assay, and lymphocyte transformation assay using glucose consumption test.

Experimental

Fresh sheep blood was collected in sterile Alsevar's solution. Cells were adjusted by using Neubaur chamber for challenge. A total of 27 male Wistar albino rats (150 - 200 g), 27 native rabbits (2-2.5 Kg) and 27 Ross broiler chicks. (2-2.5 Kg) were obtained. The rats were housed in cages (35 x 35 x 15 cm for 3-5 rats, 2-3 chicks and one rabbit). The animals received *ad libitum* a commercial diet obtained from General Company of Feed Silo and Powder Mint for rats and chicks and a commercial pelleted ration for rabbits. The diet was formulated by NRC[20]. The NIH Guide for the Care and Use of Laboratory Animals was followed throughout the experimental.

The methanolic extract of aerial parts of the *C. spinosa* plants during flowering season were collected and subjected to qualitative and quantitative analysis to identify the presence of the alkaloids, flavonoids, tannins, phenols and saponins[21].GC/MS analysis of methanolic extract was performed using Agilent Gas Chromatography [22](Model 6890N coupled to 5973 Mass Selective Detector (MSD), (USA).

Animals in each species were divided into three equal groups. Challenge of the first group was performed at days zero and 15[23] by intraperitoneal injection of 0.5,2 and 4 ml of SRBCs suspension containing $1\times10^8/mm^3$ cells for rat, rabbit and chicken respectively [24-26]. The second group was injected intra peritoneally by CY monohydrate (Sigma-Aldrich) at a dose of 90 mg/kg,65 mg/kg for rat and rabbit respectively[18,27], and 3 mg/bird/day for chicken[28] for three successive days. Challenge with SRBCs started in day 4 and day 18 as the same regimen of the first group. The third group received 30 mg/kg body weight/day of *C. spinosa* extract suspended in 1.0 ml saline for 2 weeks followed by SRBCs challenge similar to the treatment of the first group.

Blood samples were taken before challenge or at the fourth day of CY injection or after two weeks of *C. spinose* extract (day 0) and on days 7, 14, 21, 28, 35 and 42 post- challenge. The sera were obtained and inactivated at 56°C for 30 min to remove nonspecific agglutinins. Part of blood samples was heparinized for counting TLC[29] as well as N %, L % and N/L ratio [30]. For chickens, TLC was carried out using Natt-Herrick's staining solution.

Spleens were gently removed after 42 days of challenge and each was divided into two halves. The first half was used for determination of viable cell number by the trypan blue dye exclusion method[31]. The other half was rapidly preserved in 10% aqueous neutral buffered formalin solution for histopathological examination[32]**.**

ELISA was used to measure total Igs, IgM and IgG anti-SRBC membrane antibody titers in sera[33-34]**.** Hemoglobin-free SRBC membranes were prepared and the protein content of the solution was measured using SPECTRUM kits**.** ELISA plates were coated with prepared SRBC membrane and non-specific binding sites were blocked with 1% skim milk[23]. Each serum sample was then added, incubated overnight at 4°C. Enzyme

conjugate horseradish peroxidase labeled goat anti-rabbit IgG at a titer of 1:3000 for rabbit samples or labeled goat anti – mouse IgG fraction at a titer of 1:7000 dilution for rat samples or labeled goat anti - chicken IgG fraction at a titer of 1:2000 dilution for chicken samples in PBS, PH 7.2 were then added to wells. Enzyme substrate solution containing Orthophenyldiamine (OPD, Sigma-Aldrich) was added followed by peroxidase substrate and the reaction was stopped with H_2SO_4 2.5M. The color reaction was measured at wavelength 450 nm using microplate ELISA reader. The use of mercaptoethanol-resistant IgG and sensitive IgM was followed as previously described[35].

In vitro carbon clearance assay was done by collecting1.5 ml of heparinised blood samples on day 42 of treatment. Each sample was mixed with 6 µl of India ink (Pelikan4001, Brillant-Schwarz) and then divided into three equal parts for incubation at 37°C for 20 and 40 min. 150 µl of each mixture was added to 2 ml saline following incubation. The diluted samples were centrifuged at 2500 rpm for 5 min and the optical density of supernatant was read at 535 nm by spectrophotometer. Phagocytic index was calculated by converting the readings to log2 / hour [36].

For*in vivo* carbon clearance assay**,** each animal was intravenously injected with 10 ml/kg body weight of Indian ink mixtureon day 42 of challenge. Blood samples were collected after 2 and 10 min later on 4ml of 0.1% sodium carbonate solution to lyses the erythrocytes. Absorbance was measured at 670 nm using spectrophotometer. Rate of carbon clearance (K) and phagocytic index were calculated using the formula[37-38]:

$K = \log OD1 - \log OD2 / t2 - t1$

Where OD1 and OD2 are the optical densities at time t1 and t2, respectively.

Glucose consumption was measured to evaluate lymphocyte transformation [39]. Whole blood (200 µl) was mixed with 2 ml of .1% sodium carbonate solution, incubated for a short time followed by centrifugation. The cells were washed twice with PBS and twice with the RPMI- 1640 culture medium. Lymphocytes at a concentration of 2×10^6/200µl were cultured in the presence of 5 µg/ml Phytohaemagglutinin-P (PHA, Sigma-Aldrich) in 24-well plates for T cell mitogen. The cultures were kept in CO2 incubator for 72 hours. Glucose was estimated in incubation medium at 500 nm using SPECTRUM kits. The Glucose consumption was estimated as the quantity of glucose (mg/dl) consumed minus the concentration at the beginning.

Statistical analysis:

Values of data were illustrated as means ± standard errors. Statistical analysis was performed using ANOVA followed by Duncan's Multiple Range Test with $P<0.05$ being considered statistically significant[40]. Statistical analysis was conducted with SAS program[41].

Results

Table (1) illustrated the compounds detected by area from *C. spinosa* extract. Table (2) showed TLC, N%, L% and N/L in rats, rabbits and chickens at days zero, 14 and 42 pretreated with CY and *C. spinosa* after challenged with SRBC. At day zero, TLC in rats pretreated with CY was significantly decreased when compared with challenged rats ($P<0.05$). Results of N% andL% of challenged rats and exposed to CY showed that at days zero, 14 and 42 there was a significant decrease when compared with SRBC challenged group($P<0.05$). At days zero and 42 of challenge, N/L of CY pretreated group was significantly decreased than that of SRBC challenged rats ($P<0.05$). At day zero and 14, TLC of challenged rabbits pretreated with CY was significantly lower than that of SRBC challenged group ($P<0.05$). Results in the day 14 of challenge indicated that CY pretreated group showed significantly lowered N% than SRBC challenged rabbits ($P<0.05$). Concerning the L% in rabbits at day zero, 14 and 42 post challenge, the results revealed that challenged rabbits and pretreated with CY showed significant drop of L % than SRBC challenged group ($P<0.05$). There was no significant change in N/L by CY pretreated group challenged with SRBC ($P<0.05$).

Results revealed that at day zero and 42, TLC of chickens pretreated with CY was significantly lower than in challenged group with SRBC ($P<0.05$). There was no significant change in heterophil (H%) and H/L in challenged group and pretreated with CY ($P<0.05$).At days zero,14 and 42, CY pretreated group showed a significant lower L % than SRBC challenged chickens.

Table (1): Compounds detected by area from *C. spinosa* methanolic extract:

Name of the compounds	% Area
Octadecanoic acid	30.22
8,11,14-Eicosatrienoic acid	24. 4
Phytol	24.04
Farnesol	22.8
Hexadecanoic acid	22.3
Megastigmatrienone	17.9
1-hexadecene	17.7
4-methyl-2,5-dimethoxybenzaldehyde	17.6
2-Undecene	15.9
4-vinyl-2-methoxy-phenol	14.0
1,2,3-propanetriol	3.74

Table (2): Total white blood cell counts (TLC 10^3/mm³). Neutrophil (N %) Lymphocyte (L %) of animals challenged with SRBC and pretreated with CY and*C. spinosa*

Groups	Parameters	Rats			Rabbits			Chickens		
		0 Day	Day 14	Day 42	0 Day	Day 14	Day 42	0 Day	Day 14	Day 42
SRBC	**TLC 10^3/mm³**	6.51 ±0.14^{b}	6.53 ±0.15^{cd}	5.96 ±0.098^{b}	6.47 ±0.22^{bc}	7.09 ±0.15^{a}	6.12 ±0.17^{d}	11.37 ±0.31^{e}	10.87 ±0.58^{d}	13.55 ±0.917^{c}
CY + SRBC		5.12 ±0.09^{c}	6.43 ±0.35^{cd}	5.82 ±0.17^{b}	5.10 ±0.11^{d}	5.91 ±0.22^{b}	5.99 ±0.42^{d}	9.40 ±0.21^{f}	10.66 ±0.26^{d}	10.91 ±0.33^{d}
C. *spinosa*+ SRBC		6.56 ±0.19^{b}	7.12 ±0.09^{ab}	7.22 ±0.11^{a}	6.99 ±0.49^{ab}	7.46 ±0.39^{a}	7.42 ±0.49^{abc}	17.25 ±0.067^{b}	17.35 ±0.12^{b}	16.45 ±0.15^{b}
SRBC	**N %**	50.82 ±0.36^{b}	52.79 ±0.16^{b}	54.71 ±0.17^{b}	51.58 ±0.12^{b}	54.38 ±0.12^{bc}	55.76 ±0.14^{bc}	32.54 ±0.13^{de}	33.75 ±0.09^{bc}	36.56 ±0.15^{b}
CY + SRBC		34.42 ±0.18^{c}	38.71 ±2.37^{c}	42.65 ±2.38^{c}	48.42 ±2.45^{b}	45.76 ±2.33^{d}	50.14 ±0.09^{cd}	32.01 ±0.56^{de}	33.61 ±3.13^{bc}	35.57 ±0.86^{b}
C. *spinosa*+ SRBC		62.56 ±2.46^{a}	62.76 ±4.05^{a}	65.82 ±3.73^{a}	58.25 ±2.54^{a}	59.39 ±4.53^{ab}	64.49 ±3.89^{a}	39.05 ±1.41^{a}	38.69 ±0.12^{a}	38.61 ±0.16^{a}
SRBC	**L %**	50.33 ±0.10^{b}	55.48 ±0.15^{a}	58.01 ±0.93^{a}	29.78 ±0.32^{d}	30.61 ±0.11^{c}	31.61 ±0.11^{d}	48.30 ±0.17^{c}	44.93 ±0.19^{d}	49.86 ±0.13^{ab}
CY + SRBC		34.51 ±0.14^{c}	49.41 ±2.54^{bc}	42.38 ±1.98^{c}	21.01 ±0.21^{e}	22.84 ±0.23^{e}	23.58 ±0.20^{e}	42.96 ±0.25^{d}	48.51 ±0.19^{c}	53.65 ±2.41^{a}
C. *spinosa*+ SRBC		52.42 ±2.49^{ab}	53.56 ±0.15^{abc}	54.66 ±0.11^{b}	57.00 ±0.09^{a}	55.49 ±0.17^{a}	54.87 ±0.24^{a}	55.80 ±1.90^{b}	57.16 ±0.13^{a}	53.31 ±0.14^{a}
SRBC	**N/L**	1.48 ±0.15b	1.69 ±0.12a	1.41 ±0.13a	1.54 ±0.14a	1.51 ±0.13a	1.47 ±0.16a	0.6 ±0.11b	0.64 ±0.09b	0.62 ±0.12a
CY + SRBC		0.84 ±0.05c	1.56 ±0.14a	0.81 ±0.05b	1.56 ±0.13a	1.46 ±0.12a	1.55 ±0.09a	0.65 ±0.11b	0.66 ±0.14b	0.57 ±0.12a
C. *spinosa*+ SRBC		1.62 ±0.09b	1.48 ±0.13a	1.41 ±0.15a	1.52 ±0.15a	1.47 ±0.14a	1.66 ±0.11a	0.60 ±0.10b	0.72 ±0.09b	0.64 ±0.11a

Means having different letters in the same column within the same parameter and animal are significantly different (P<0.05).

Concerning days 14 and 42 as shown in table (2) TLC and N % of challenged rats pretreated with *C. spinosa* were significantly higher while L% was significantly decreased than challenged rats (P<0.05). No significant change in N/L between rats pretreated with*C. Spinosa* and the challenged group. At day 42, TLC of rabbits pretreated with *C. spinosa* were significantly increased compared with rabbits challenged with SRBC. Results at day zero and 14 revealed that rabbits pretreatedwith *C. spinosa* showed significantly higher N% than

SRBC challenged rabbits (P<0.05). TLC and H% in challenged chickens pretreated with *C. spinosa* were significantly increased than TLC in chickens challenged with SRBC at all sampling days.

Concerning spleen cellular viability % of rats, rabbits and chickens at day 42 post challenge, the results in table (3) shown that CY pretreated rats group was significantly decreased than that of the SRBC challenged animals (P<0.05). *C. spinosa* pretreated groups of rats and rabbits showed significant lower spleen cellular viability % than that of the SRBC challenged animals(P<0.05).

Table (3): Spleen cellular viability % of animals at day 42 of animals challenged with SRBC and pretreated with CY and *C. spinosa*.

Means having different letters in the same column within the same animal are significantly different (P<0.05).

Results in table (4) showed *in vivo* and *in vitro* phagocytic index in rats, rabbits and chickens pretreated with CY or*C. Spinosa* at day 42 of challenge. In rats and rabbits, the phagocytic index of CY pretreated group was significantly decreased than that of. SRBC challenged group (P<0.05). *C. spinosa* pretreated rats and chickens showed a significant higher values than that of SRBC challenged group (P<0.05). However, the phagocytic index of *C. spinosa* pretreated rabbits was significantly decreased comparedto SRBC challenged group.

Groups	*In vitro* phagocytic index			*In vivo* phagocytic index		
	Rats	Rabbit	Chickens	Rats	Rabbit	Chickens
SRBC	0.0061 ±0.0002^{c}	0.0065 ±0.00002^{d}	0.0033 ±0.00002^{d}	0.0057 ±0.00005^{d}	0.0064 ±0.00006^{d}	0.0033 ±0.00001^{d}
CY + SRBC	0.0009 ±0.0002^{e}	0.0015 ±0.00007^{g}	0.0033 ±0.00002^{d}	0.0013 ±0.00007^{f}	0.0015 ±0.00006^{g}	0.0033 ±0.00003^{d}
C. *spinosa*+ SRBC	0.0108 ±0.0001^{b}	0.0051 ±0.00004^{f}	0.0137 ±0.00002^{a}	0.0109 ±0.00006^{b}	0.0089 ±0.00002^{c}	0.0137 ±0.00003^{a}

Table (4): *In vitro and in vivo* carbon clearance test in animals challenged with SRBC and pretreated with CY and *C. spinosa*.

Groups	Rats	Rabbits	chickens
SRBC	99.41±0.13a	98.44±0.16a	91.57±0.09b
CY + SRBC	88.50±0.08e	85.49±0.12e	86.46±0.16c
***C. spinosa* + SRBC**	95.56±0.13b	94.81±2.21b	91.51±1.90b

Means having different letters in the same column within the same parameter and animalare significantly different (P<0.05).

Table (5): Glucose consumption (mg/dl) of blood lymphocytes stimulated by PHA in animals challenged with SRBC and pretreated with CY and *C. spinosa*.

Groups	Glucose concentration in the medium (mg/dl) after 3 days of incubation.								
	Rats			Rabbits			Chickens		
	Without PHA	With PHA	Glucose consumption	Without PHA	With PHA	Glucose consumption	Without PHA	With PHA	Glucose consumption
SRBC	40.68 ±0.22^{e}	38.75 ±0.34^{c}	1.92 ±0.25^{d}	40.67 ±0.22^{e}	38.89 ±0.29^{d}	1.78 ±0.15^{d}	41.63 ±0.17^{d}	38.73 ±0.23^{c}	2.90 ±0.14^{e}
CY + SRBC	41.90 ±0.29^{d}	40.77 ±0.34^{b}	1.12 ±0.11^{e}	41.72 ±0.18^{d}	40.89 ±0.37^{b}	0.82 ±0.21^{e}	42.34 ±0.19^{bcd}	40.67 ±0.19^{b}	1.67 ±0.13^{f}
C. *spinosa*+ SRBC	44.87 ±0.24^{a}	36.85 ±0.33^{d}	8.02 ±0.11^{a}	44.79 ±0.28^{a}	36.88 ±0.31^{e}	7.91 ±0.14^{a}	43.70 ±0.27^{a}	36.80 ±0.34^{d}	6.90 ±0.15^{b}

Means having different letters in the same column within the same parameter and animal are significantly different (P<0.05).

The results of glucose consumption of PHA stimulated blood lymphocytes of CY pretreated animals is presented in table (5) It shows significant decrease than that of SRBC challenged groups (P<0.05). *C. spinosa* pretreated animals showed higher significant values than that of SRBC challenged rats(P<0.05).

Table (6): Total anti-SRBC antibodies (Igs) in serum of animals challenged with SRBC and pretreated with CY and*C. spinosa*.

Groups	Animals	Days post challenge						
		0	**7**	**14**	**21**	**28**	**35**	**42**
SRBC	**Rats**	0.49 ±0.07ab	0.53 ±0.06a	0.53 ±0.05a	0.53 ±0.09ab	0.47 ±0.07ab	0.49 ±0.05ab	0.46 ±0.05a
CY+ SRBC		0.23 ±0.048b	0.45 ±0.13a	0.34 ±0.07a	0.28 ±0.04bc	0.32 ±0.07b	0.30 ±0.07b	0.43 ±0.08a
C. *spinosa*+ SRBC		0.59 ±0.12a	0.67 ±0.07a	0.63 ±0.13a	0.64 ±0.12a	0.66 ±0.07a	0.69 ±0.12a	0.68 ±0.09a
SRBC	**Rabbits**	0.41 ±0.05ab	0.64 ±0.10a	0.54 ±0.12ab	0.54 ±0.11ab	0.49 ±0.09a	0.49 ±0.14ab	0.69 ±0.09a
CY+ SRBC		0.34 ±0.13ab	0.23 ±0.04c	0.19 ±0.02c	0.21 ±0.03b	0.17 ±0.02b	0.31 ±0.13ab	0.15 ±0.03c
C. *spinosa*+ SRBC		0.50 ±0.10ab	0.53 ±0.13ab	0.50 ±0.12ab	0.64 ±0.13a	0.71 ±0.07a	0.59 ±0.14ab	0.57 ±0.13ab
SRBC	**Chickens**	0.35 ±0.07ab	0.64 ±0.02 ab	0.74 ±0.07a	0.76 ±0.05a	0.70 ±0.08ab	0.81 ±0.06a	0.51 ±0.04 ab
CY+ SRBC		0.26 ± 0.06b	0.31 ±0.08cd	0.38 ±0.06b	0.40 ±0.081b	0.45 ±0.09bc	0.34 ±0.08c	0.27 ±0.06b
C. *spinosa*+ SRBC		0.42 ±0.12 ab	0.69 ±0.09a	0.76 ±0.06a	0.76 ±0.05a	0.80 ±0.05a	0.73 ±0.05 ab	0.39 ±0.09 ab

Means having different letters in the same column within the same animals are significantly different (P<0.05).

Tables (6, 7, 8) showed the total anti-SRBC antibodies (Igs), mercaptoethanol sensitive (IgM) and mercaptoethanol resistant (IgG) antibody titers at zero, 7, 14, 21, 28, 35 and 42 days post challenge in serum of rat, rabbits and chickens pretreated with CY and *C. spinosa*. Results showed that in CY pretreated post challenged rabbits, Igs at day 7, 14, 28 and 42 days was significantly decreased than that of SRBC challenged group. In the day 7, 14, 21 and 35 post challenge, Igs in serum of CY pretreated chickens was significantly decreased than that of SRBC challenged chickens(P<0.05). IgM antibody titers at day 7 of CY treatment of post challenge was significantly lowered than that in SRBC challenged rats (P<0.05). However, at day 7, 14 and 42 post challenge. IgM in CY treated group was significantly decreased than that in SRBC challenged rabbits. It was also found that at the days 14and 35 post challenged chickens and treated with CY showed a significant decreased in IgM antibody titers than challenged group (P<0.05). Results showed that IgG antibody titers in all days post challenge and treated with CY, IgG antibody titersin serum were significantly lower than that of SRBC challenged chickens (P<0.05). There was no significant effect of *C. spinosa* treatment on Igs. As shown in results, it was found that in the days 21, 35 and 42 post challenge, IgM of *C. spinosa* pretreated rats was significantly higher than that of SRBC challenged rats. In the day 14 post challenge, IgG in serum of *C. spinosa* pretreated rabbits was significantly increased than that of SRBC challenged rabbits.

Table (7): Mercaptoethanol sensitive (IgM) antibody titers in serum of animals challenged with SRBC and pretreated with CY and *C. spinosa*.

Groups	Animals	Days post challenge						
		0	7	14	21	28	35	42
SRBC	Rats	0.062 ±0.01a	0.346 ±0.16abc	0.367 ±0.18ab	0.152 ±0.07bc	0.360 ±0.14ab	0.184 ±0.04bc	0.061 ±0.01de
CY+ SRBC		0.067 ±0.01a	0.038 ±0.01d	0.091 ±0.02b	0.093 ±0.03c	0.071 ±0.02b	0.085 ±0.03c	0.097 ±0.03cde
C. *spinosa*+ SRBC		0.062 ±0.01a	0.636 ±0.12a	0.619 ±0.11a	0.507 ±0.11a	0.546 ±0.13a	0.495 ±0.12a	0.712 ±0.12a
SRBC	Rabbits	0.066 ±0.01a	0.589 ±0.14a	0.513 ±0.14a	0.474 ±0.11ab	0.480 ±0.12ab	0.454 ±0.16abc	0.514 ±0.14a
CY+ SRBC		0.052 ±0.01a	0.115 ±0.02bc	0.135 ±0.02b	0.169 ±0.02bc	0.147 ±0.01b	0.160 ±0.02bc	0.112 ±0.05bc
C. spinosa+ SRBC		0.041 ±0.01a	0.468 ±0.15ab	0.560 ±0.10a	0.506 ±0.11a	0.533 ±0.12a	0.501 ±0.12ab	0.473 ±0.15ab
SRBC	Chickens	0.040 ±0.02a	0.329 ±0.14ab	0.405 ±0.08a	0.457 ±0.15ab	0.292 ±0.09ab	0.606 ±0.12a	0.373 ±0.10ab
CY+ SRBC		0.057 ±0.01a	0.096 ±0.02b	0.067 ±0.01b	0.097 ±0.04bc	0.133 ±0.04b	0.157 ±0.06bc	0.071 ±0.01b
C. spinosa+ SRBC		0.052 ±0.02a	0.358 ±0.11ab	0.468 ±0.15a	0.499 ±0.15a	0.621 ±0.12a	0.342 ±0.09abc	0.536 ±0.14a

Means having different letters in the same column within the same animalsare significantly different (P<0.05).

Table (8): Mercaptoethanol resistant (IgG) antibody titers in serum of animals challenged with SRBC and pretreated with CY and *C. spinosa*.

Groups	Animals	Days post challenge						
		0	7	14	21	28	35	42
SRBC	Rats	0.149 ±0.01a	0.444 ±0.06abc	0.356 ±0.03ab	0.316 ±0.05bcd	0.386 ±0.06ab	0.382 ±0.05abc	0.295 ±0.05bc
CY+ SRBC		0.138 ±0.02a	0.202 ±0.06c	0.280 ±0.12bc	0.131 ±0.039d	0.108 ±0.05b	0.180 ±0.05bc	0.144 ±0.01c
C. spinosa + SRBC		0.146 ±0.01a	0.590 ±0.08ab	0.615 ±0.14a	0.624 ±0.07a	0.485 ±0.12a	0.602 ±0.14a	0.579 ±0.07a
SRBC	Rabbits	0.083 ±0.02a	0.630 ±0.12ab	0.308 ±0.05bc	0.389 ±0.09abc	0.504 ±0.04a	0.556 ±0.04a	0.584 ±0.12a
CY+ SRBC		0.076 ±0.02a	0.083 ±0.02c	0.101 ±0.03c	0.126 ±0.05c	0.061 ±0.01b	0.074 ±0.01b	0.132 ±0.04b
C. spinosa+ SRBC		0.090 ±0.03a	0.416 ±0.07b	0.591 ±0.08a	0.542 ±0.10ab	0.467 ±0.12a	0.485 ±0.07a	0.665 ±0.09a
SRBC	Chickens	0.083 ±0.02a	0.630 ±0.12ab	0.308 ±0.05bc	0.389 ±0.09abc	0.504 ±0.04a	0.556 ±0.04a	0.584 ±0.12a
CY+ SRBC		0.111 ±0.04a	0.126 ±0.03ab	0.084 ±0.02c	0.242 ±0.09bc	0.092 ±0.02b	0.109 ±0.01b	0.105 ±0.03b
C. spinosa+ SRBC		0.082 ±0.02a	0.533 ±0.14c	0.515 ±0.14ab	0.477 ±0.12ab	0.529 ±0.14a	0.478 ±0.16a	0.564 ±0.16a

Means having different letters in the same column within the same animals are significantly different (P<0.05).

The microscopic examination of rats challenged with SEBCs revealed mild immunopromotion characterized by slight enlargement of the white pulps (Fig. 1A). Upon pretreatment with *C. spinosa* extract (Fig. 1B), more improvement of the immunostimulant criteria of the spleen was noticed as evidenced by lymphoid cell hyperplasia with subsequent hypertrophy of the splenic white pulps or follicles. As it was expected, pretreatment of CY caused marked relative immunodepletion characterized microscopically by the

classic moth-eaten appearance of the splenic parenchyma as well as follicular atrophy (Figs. 1C). Regarding rabbits, the above-mentioned tissue reactions associated with SEBCs challenge alone or in combination with pretreatment with *C. spinosa* extract were evident in similar patterns. (Fig. 2 D, E, F).

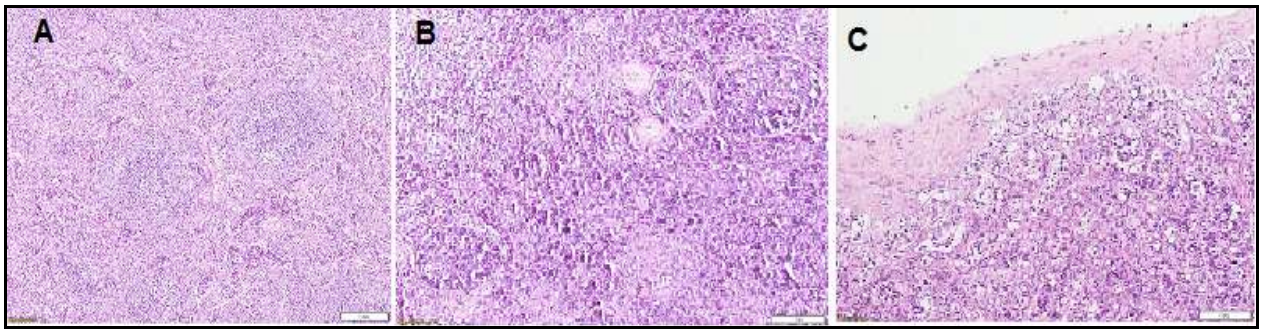

Fig. (1): (A) Spleen of a rat challenged with SRBCs showing slight enlargement of the white pulps. H&E. X 160. (B)Spleen of a challenged rat and pretreated with *C. spinosa* extract showing follicular lymphoid cell hyperplasia and hypertrophy. H&E. X160.(C) Spleen of achallenged rat and exposed to CY showing severe lymphoid cell hyperplasia, necrosis and depletion giving moth-eaten appearance. H&E. X 250.

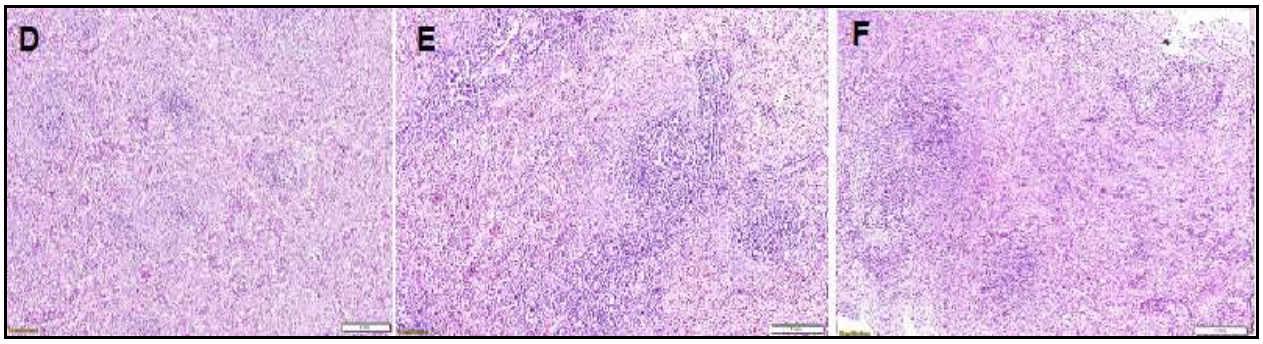

Fig. (2):(D) Spleen of a rabbit challenged with SRBCs showing lymphoid cell hyperplasia. H&E. X 160. (E)Spleen of a challenged rabbit and pretreated with *C. spinosa* extract showing enlargement of the splenic white pulps. H&E. X 160. (F) Spleen of a rabbit received CY showing follicular atrophy. H&E. X160.

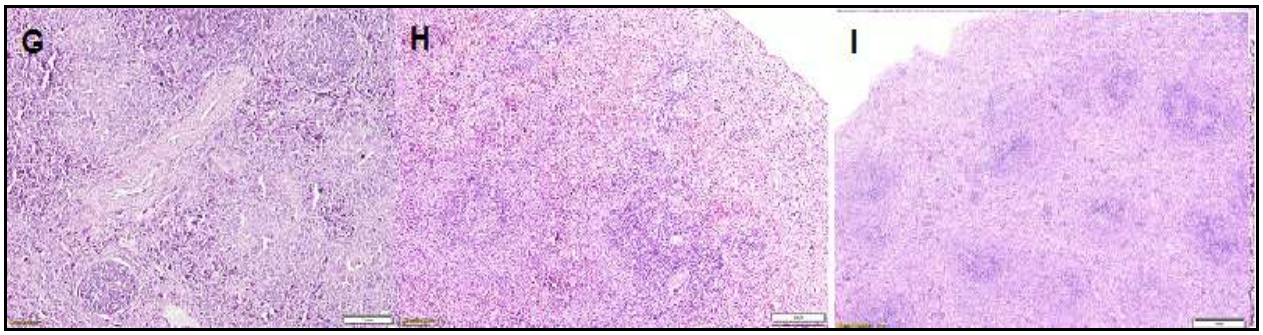

Fig. (3): (G) Spleen of a chicken challenged with SRBCs showing slight enlargement of the white pulps. H&E. X 160. (H)Spleen of a chicken given *C. spinosa* extract showing hypertrophy of the white pulps. H&E. X 160. (I) Spleen of a chicken given CY showing severe atrophy of the white pulps. H&E. X 160.

In case of chicken, injection of SRBCs resulted in some degree of immunostimulation where in the splenic lymphoid cell population increased in number, so that the splenic follicles appeared slightly enlarged (Fig. 3G). When such cases were pre- treatment with *C. spinosa*, there were marked increases in number of the lymphoid cells resulted in higher cellular density besides enlargement of the splenic follicles at the expense of the size of the red pulp regions (Fig. 3H). Moreover, lymphoid cells were seen interspersed throughout these red pulp regions. With regard to pretreatment of challenged chickens with CY, the fore mentioned parenchymal

moth-eaten appearance was noticed due to severe diffuse lymphoid cell depletion (Figs. 3I). Additionally, the affected white pulps showed severe atrophy.

Discussion

Many scientists are turning to cheap counting of white blood cells from blood films to assess immune status in animals [42]. Blood samples were collected in this study prior to challenge with SRBCs or on the fourth day of CY injection or after two weeks administration of *C. spinosa* extract (day 0) and at days 7, 14, 21, 28, 35 and 42 post-challenge. Part of blood samples were used for counting the TLC as well as N %, L % and N/L ratio.

Results of TLC, N% andL% of challenged rats and exposed to CY were significantly decreased than SRBC challenged group. This data is in agreement with [17, 43] who reported that CY is an ideal immune suppressive agent. CY suppressed the immune system through myelosuppression and lymphocytes toxicity via interfering with nucleic acid function. The mechanism of action of CY was mentioned by Hosseinimehr et al. [44] who reported that CY produced biologically active metabolites by microsomal enzymes that caused chromosomal damage through the generation of free radicals. CY was able to induce immunosuppression in animal models [45]. It depressed also both types of immunity by acting on B and T lymphocytes[18].

N/L of CY pre-challenged groups differ in species used. It either significantly lowers than SRBC challenged rats or not affected in rabbits and chickens. Many studies focused on the N/L ratio. Higher ratio indicated that the animal used its innate immunity while low ratio indicated that the animal used the acquired immune system[46].

Concerning spleen cellular viability % of rats, rabbits and chickens at day 42 post challenge, the results revealed that CY pretreated rats were decreased compared with the SRBC challenged animals. This data is in agreement with Nitharwal et al.[19] who indicated apoptosis effect of CY on splenocytes and lymphocytes. The drug also caused inhibition of lymphocyte proliferation, also, there was a modulation of the expressions of gene sand transcription factors.

TLC and N % of challenged rats and rabbits pretreated with *C. spinosa* were significantly increased while L% was significantly decreased than challenged group. No significant changes in N/L between rats pretreated with *C. Spinosa* and challenged groups. TLC and H% in challenged chickens pretreated with *C. spinosa* were significantly increased than TLC in challenged chickens with SRBC in all sampling days.*C. spinosa* pretreated groups of rats and rabbits showed significantly lower spleen cellular viability % than that of the SRBC challenged animals. These results indicated amelioration of leucocyte count as previously reported[15] who suggested that *C. spinosa* ethanol extracts might be used to treat inflammatory diseases and considered as immunomodulatory through maturation of mouse dendritic cells (DCs) derived from bone marrow and also restored the myelosuppressive effects induced by CY.

Cellular immune response was performed using phagocytosis index of *in vitro* and *in vivo* carbon clearance assay and lymphocyte transformation assay using glucose consumption test. Assay of phagocytic index by the clearance of carbon was aimed to evaluate the effect of *C. spinosa* extracts or CY on the immune system that contains the phagocytic cells. When carbon particles are injected into the animals, the rate of clearance of carbon from the blood by phagocytic cells is converted to phagocytosis index by equation[47]. Neutrophils are one of the professional phagocytic cells that play a critical role in both innate and adaptive immune response [48]. Results indicated that, in rats and rabbits, the phagocytic index of CY pretreated group were significantly decreased than that of SRBC challenged group. *C. spinosa* pretreated rats and chickens showed significant higher values. These results indicated that pretreatment of animals with extract induced augmentation of the phagocytic function **[4].** Results of the current study on the glucose consumption of PHA stimulated blood lymphocytes were parallel to the results obtained on phagocytic index.

Concerning humoral immune responses of rats, rabbits and chickens pretreated with CY or *C. spinosa* extract, we focused on assessment of production of Igs, IgM and igG antibodies. The solid phase ELISA using sheep red blood cells membrane as antigen was performed for estimation. Results showed that CY pretreated animals exhibited lower Igs, IgG and IgM antibodies titers than that of SRBC challenged group. It was found that IgM and IgG antibody titers of *C. spinosa* pretreated rats increased significantly than that of SRBC

challenged rats without changes in Igs. The augmentation of the response was previously reported by Miller [49] who indicated enhanced responsiveness of T and B lymphocyte lineages that participated in the antibody production. Recently, many studies reported that *C. spinosa* contains a plenty of biochemical compounds including flavonoids, alkaloids, polyphenols, saponins, terpenoids, lectin, essential oils, glycosinolate, and glycosides[50-51], which exhibited activities such as anti-inflammatory[52] and immunomodulatory[12]**.**It is interesting to illustrate that ethanol extract of *C. spinosa* have demonstrated to stimulate both humoral and cell mediated immune responses[53]. The effect produced by *C. spinosa* in in this study can be due to its flavonoids and octadecanoic acid (30.22 %), which are known to possess immunomodulatory properties.

The immune response based upon the microscopic findings in the spleen induced by the tested materials, regardless of the used species, was noticed in comparison with the control negative group. The challenge with SRBC in the different species induced mild degree of immuno stimulation. A prominent immunostimulation was noticed in case of *C. spinosa*. On the other hand, the unique immunodepressive effect was noticed following the use of CY.

In conclusion, pretreatment of animals with *C. spinosa* extract for two weeks enhanced both cellular and humoral immune responses. In addition, such findings were confirmed by histological studies. Results of this study may encourage the use of *C. spinosa* extract in lowering the immunotoxicity of CY in animal models. In addition, results expected from this may pave the road for further immune improvement for better healthy state.

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